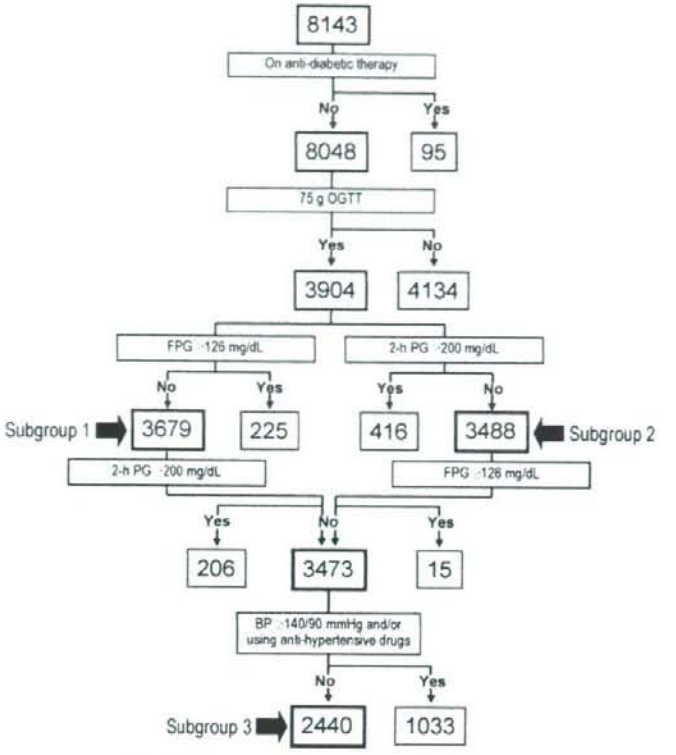

Fig. 1. Flow chart showing selection of the four subgroups.

2. Methods

2.1. Study subjects and selection of subgroups

The study was approved by The Ethical Committee of Mitsui Memorial Hospital and University of Tokyo, Faculty of Medicine. Between September 1994 and December 2003, 8143 subjects underwent general health screening including carotid ultrasonography at the Center for Multiphasic Health Testing and Services, Mitsui Memorial Hospital. Of the 8143 subjects, 95 were treated as having diabetes, and of the remaining 8048 individuals, 3904 underwent an OGTT. Among these 3904 individuals, three subgroups were sequentially selected based on various parameters (Fig. 1). Those with a fasting plasma glucose (FPG) value of <126 mg/dL were designated as subgroup 1, and those with a 2-h post-OGTT plasma glucose (2-h PG) value of <200 mg/dL were designated as subgroup 2. Subgroup 3 was comprised of subjects who met all the following conditions: FPG of <126 mg/dL, 2-h PG of <200 mg/dL, and not having hypertension. Hypertension was defined as SBP ≥140 mmHg, DBP ≥90 mmHg, or the use of antihypertensive medication. We also selected individuals without impaired glucose tolerance (IGT), i.e., individuals with a 2-h PG value of <140 mg/dL.

At our institute, several types of health screening programs are available, and some general health screening programs include carotid ultrasonography and/or OGTT, while others do not. However, the decision on the type of health screening was made by the individuals and/or their companies and was not decided upon or recommended by any attending physician.

2.2. Definition of MetS

MetS was defined as the presence of three or more of the following: (1) fasting glucose ≥110 mg/dL; (2) SBP/DBP ≥130/85 mmHg or taking anti-hypertensive medication; (3) triglycerides ≥150 mg/dL mmol/L; (4) HDL cholesterol <40 mg/dL in men and <50 mg/dL in women; and (5) body mass index ≥25 kg/m^2 [11].

2.3. Carotid ultrasonography

Carotid artery status was studied using high resolution B-mode ultrasonography (Sonolayer SSA270A, Toshiba, Japan) equipped with a 7.5 MHz transducer as described previously [12]. Plaque was defined to be present when there is one or more clearly isolated focal thickening(s) of the intima-media layer with thickness of ≥1.3 mm at the common or internal carotid artery or the carotid bulb. Carotid wall intima-media thickening was said to be present when intima-media thickness which was measured at the far wall of the distal 10 mm of the common carotid artery was ≥1.0 mm [12].

2.4. Statistical analysis

Logistic regression analysis was used to obtain adjusted odds ratios and their 95% confidence intervals (CIs) to predict the presence of carotid plaque or carotid intima-media thickening. Statistical analyses were carried out by using Dr. SPSS II (SPSS Inc., Chicago, IL). Results are expressed as the mean ± standard deviation (SD). A value of $p < 0.05$ was taken to be statistically significant.

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Table 1
Baseline characteristics.

Variables	Subgroup 1		Subgroup 2		Subgroup 3	
	Men	Women	Men	Women	Men	Women
Number	2548	1131	2386	1102	1588	852
Age, years	58.2 ± 10.6	57.9 ± 10.4	58.0 ± 10.7	57.8 ± 10.3	56.7 ± 10.9	56.6 ± 10.5
Body mass index, kg/m^2	24.0 ± 2.8	22.2 ± 3.1	23.9 ± 2.7	22.1 ± 3.1	23.6 ± 2.6	21.7 ± 2.8
Systolic BP, mmHg	127 ± 19	121 ± 21	128 ± 19	120 ± 20	119 ± 12	123 ± 14
Diastolic BP, mmHg	79 ± 12	73 ± 12	79 ± 12	73 ± 12	73 ± 8	69 ± 9
Total cholesterol, mg/dL	206 ± 32	219 ± 35	205 ± 32	219 ± 35	205 ± 32	216 ± 35
HDL-cholesterol, mg/dL	55 ± 16	70 ± 17	55 ± 16	70 ± 17	56 ± 16	71 ± 17
Triglycerides, mg/dL	144 ± 117	96 ± 56	142 ± 98	95 ± 54	141 ± 98	95 ± 54
Uric acid, mg/dL	6.2 ± 1.2	4.7 ± 1.0	6.2 ± 1.2	4.7 ± 1.0	6.2 ± 1.2	4.6 ± 1.0
Fasting glucose, mg/dL	96 ± 10	90 ± 10	95 ± 10	90 ± 9	94 ± 9	88 ± 9
2-h OGTT glucose, mg/dL	132 ± 41	118 ± 32	125 ± 29	115 ± 26	121 ± 29	112 ± 25
Haemoglobin A1C, %	5.2 ± 0.4	5.1 ± 0.4	5.2 ± 0.4	5.1 ± 0.4	5.2 ± 0.4	5.1 ± 0.4
Hypertension, *n* (%)	863 (34)	263 (23)	788 (33)	248 (23)	0	0
Anti-hypertensive drugs, *n* (%)	336(13)	99(9)	307(13)	95(9)	0	0
Metabolic syndrome, *n* (%)	439(17)	84(7)	372(16)	72(7)	131 (8)	25(3)
Smoking status						
Never, *n* (%)	764 (30)	933 (82)	714(30)	909 (82)	465 (29)	689(81)
Former, *n* (%)	799(31)	53(5)	753 (32)	50(5)	464 (29)	44(5)
Current, *n* (%)	985 (39)	145(13)	919(39)	143(13)	659(41)	119(14)

BP indicates blood pressure, OGTT indicates oral glucose tolerance test.

3. Results

3.1. Association between MetS and carotid atherosclerosis in individuals with FPG value of <126 mg/dL (subgroup 1)

Among the 3904 individuals who underwent OGTT, 3679 (94%) had an FPG value of less than 126 mg/dL. Of these, 300 (257 men, 43 women), the FPG value was ≥110 mg/dL, thus impaired fasting glycemia (IFG), and in the remaining 3379 (2291 men, 1088 women) had an FPG value of less than 110 mg/dL (no IFG). Table 1 shows the baseline characteristics of this group according to gender. Carotid plaque was found in 823 (32%) men and 191 (17%) women and carotid intima-media thickening was found in 422 (17%) men and 122 (11%) women (Fig. 2). Age-adjusted logistic regression analysis (Model 2) showed that, in men, MetS was statistically significantly associated with carotid plaque (Table 1) and intima-media thickening (Table 2). In women, MetS tended to be associated with carotid plaque, but not with intima-media thickening after age adjustment. Similar patterns of relationships could be observed after further adjustment for total cholesterol (TC) and smoking status (Model 3). On the other hand, after full adjustment including that for components of MetS (Model 4), MetS was not significantly associated with carotid plaque or intima-media thickening in either men or women.

3.2. Association between metabolic syndrome and carotid atherosclerosis in individuals with 2-h PG value of <200 mg/dL (subgroup 2)

Among 3904 individuals who underwent OGTT, 3488 (89%) had a 2-h PG value of less than 200 mg/dL. Of these 3488 individuals 2644 (1717 men, 927 women) had a 2-h PG value of less than 140 mg/dL (no IGT) and the remaining 844 (669 men, 175 women) had a 2-h PG FPG value of ≥140 mg/dL, and thus IGT. Carotid plaque was found in 761 (32%) men and 182 (17%) women and carotid intima-media thickening was found in 378 (16%) men and 116 (11%) women. Age-adjusted logistic regression analysis (Model 2) showed that, in men, MetS was statistically significantly associated with carotid plaque (Table 2) and intima-media thickening (Table 3). In women, MetS tended to be associated with carotid plaque but not with intima-media thickening. Similar patterns of relationship could be observed after further adjustment for TC and smoking status (Model 3). On the other hand, after full adjustment that included components of MetS (Model 4), MetS was not significantly associated with carotid plaque or intima-media thickening in men or in women. There were only 15 (13 men, 2 women)

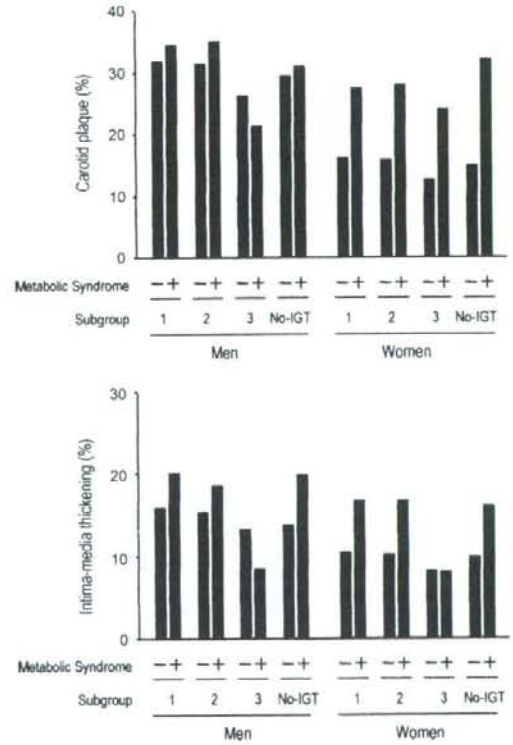

Fig. 2. Prevalence of carotid plaque and carotid intima-media thickening according to the presence or absence of metabolic syndrome in subgroups.

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Table 2
Logistic regression analysis with metabolic syndrome as an independent variable and carotid plaque as a dependent variable.

Variables	Odds ratio for carotid plaque			
	Men		Women	
	Odds ratio (95% CI)	*P* value	Odds ratio (95% CI)	*P* value
Subgroup 1				
Model 1	1.12(0.90–1.39)	0.302	1.97(1.19–3.28)	0.009
Model 2	1.41(1.11–1.79)	0.005	1.68(0.96–2.95)	0.072
Model 3	1.30(1.03–1.67)	0.030	1.63(0.93–2.88)	0.091
Model 4	1.21(0.90–1.63)	0.209	1.61(0.79–3.29)	0.188
Subgroup 2				
Model 1	1.18(0.93–1.49)	0.170	2.06(1.20–3.55)	0.009
Model 2	1.47(1.14–1.90)	0.003	1.78(0.98–3.24)	0.058
Model 3	1.38(1.07–1.78)	0.014	1.72(0.95–3.14)	0.076
Model 4	1.23(0.90–1.69)	0.202	1.73(0.82–3.63)	0.151
Subgroup 3				
Model 1	0.77(0.50–1.19)	0.232	2.20(0.86–5.62)	0.101
Model 2	0.99(0.62–1.58)	0.971	1.89(0.66–5.43)	0.235
Model 3	0.94(0.59–1.50)	0.796	1.85(0.64–5.33)	0.254
Model 4	0.82(0.48–1.41)	0.479	2.44(0.72–8.29)	0.152

Model 1, unadjusted; Model 2, adjusted for age; Model 3, adjusted for age, total cholesterol and smoking status; Model 4, adjusted for age, body mass index, systolic blood pressure, total cholesterol, HDL cholesterol, triglycerides, fasting plasma glucose, and smoking status.

individuals among the 3488 in subgroup 2 who had an FPG value of <126 mg/dL in addition to a 2-h PG value of <200 mg/dL, and, thus, the mode of association between MetS, carotid plaque, and intima-media thickening in this subgroup was essentially the same as that observed in total population of subgroup 2.

We also investigated the association between MetS and carotid atherosclerosis in individuals without IGT. There were 2644 individuals who did not have IGT, and among them, 61 had FPG value of ≥110 mg/dL (Fig. 2, Supplementary Tables 1 and 2). The obtained results in these subgroups were similar to those in the subgroup 2; however, association between MetS and carotid intima-media thickening was statistically significant even after multivariate adjustment in women.

Table 3
Logistic regression analysis with metabolic syndrome as an independent variable and carotid intima-media thickening as a dependent variable.

Variables	Odds ratio for carotid intima-media thickening			
	Men		Women	
	Odds ratio (95% CI)	*P* value	Odds ratio (95% CI)	*P* value
Subgroup 1				
Model 1	1.33(1.03–1.73)	0.031	1.74(0.95–3.19)	0.074
Model 2	1.74(1.31–2.30)	<0.001	1.40(0.72–2.73)	0.324
Model 3	1.65(1.24–2.19)	<0.001	1.38(0.70–2.70)	0.349
Model 4	0.97(0.67–1.39)	0.851	0.70(0.31–1.60)	0.398
Subgroup 2				
Model 1	1.26(0.94–1.68)	0.120	1.78(0.93–3.42)	0.083
Model 2	1.63(1.20–2.22)	0.002	1.47(0.73–2.98)	0.285
Model 3	1.55(1.13–2.11)	0.006	1.44(0.71–2.93)	0.317
Model 4	1.00(0.68–1.48)	0.993	0.71(0.30–1.67)	0.435
Subgroup 3				
Model 1	0.61(0.32–1.15)	0.125	0.99(0.23–4.28)	0.985
Model 2	0.83(0.43–1.61)	0.586	0.71(0.15–3.41)	0.673
Model 3	0.77(0.40–1.50)	0.443	0.70(0.15–3.39)	0.660
Model 4	0.52(0.24–1.11)	0.092	0.56(0.05–1.45)	0.123

Model 1, unadjusted; Model 2, adjusted for age; Model 3, adjusted for age, total cholesterol and smoking status; Model 4, adjusted for age, body mass index, systolic blood pressure, total cholesterol, HDL cholesterol, triglycerides, fasting plasma glucose, and smoking status.

3.3. Association between metabolic syndrome and carotid atherosclerosis in individuals with FPG value of <126 mg/dL, 2-h PG value of <200 mg/dL, and no hypertension (subgroup 3)

Among 3904 individuals who underwent OGTT, 2440 (63%) could be assigned to subgroups 3. Their baseline characteristics according to gender are shown in Table 1. Carotid plaque was found in 409 (26%) men and 110 (13%) women and carotid intima-media thickening was found in 202 (13%) men and 69 (8%) women. Unlike subgroups 1 and 2, MetS was not significantly associated with either carotid plaque or intima-media thickening after age adjustment, or even before any adjustment in either gender (Tables 2 and 3).

4. Discussion

Here, we have assessed whether MetS is a risk factor for carotid atherosclerosis in individuals who were determined not to have diabetes mellitus based on results of OGTT. MetS was found to be associated with carotid atherosclerosis especially in men; however, when individuals with hypertension, defined as those having SBP/DBP ≥140/90 mmHg or using anti-hypertensive medication, were excluded, the presence of MetS no longer conferred excess risk when adjustments were made only for age or even when no adjustments were made.

It is known that clustering of certain metabolic abnormalities and hypertension increases the incidence of atherosclerotic diseases [13]. However, whether such clustering of atherogenic risk factors should be separately designated as MetS has been controversial. Whether MetS is independently associated with carotid atherosclerosis has been analyzed in various populations. By analyzing data on a multi-ethnic cohort of apparently healthy individuals in Canada, Paras et al. reported that although MetS was significantly associated with measures of sub-clinical carotid atherosclerosis, this association is mediated entirely through the components of MetS that have been considered as risk factors [14]. Similarly, by analyzing data on individuals recruited from a local community in Italy, Fadini et al. demonstrated that the clustering of MetS components led to a no-more-than additive increase in carotid intima-media thickness [4]. In addition, Vaidya et al. reported that MetS did not have supra-additive association with carotid intima-media thickening [15].

In our previous study that analyzed data on subjects who underwent general health screening, we found that MetS may not be associated with carotid atherosclerosis even after adjustment only for age when individuals did not have hypertension (SBP/DBP <140/90 mmHg and not using anti-hypertensive medication) [8]. In the current study, we expanded this theme to investigate whether MetS increases the risk for carotid atherosclerosis in individuals who had no or only mild (i.e., not in the diabetic range) abnormalities in glucose metabolism. We found that in individuals with FPG values of <126 mg/dL (subgroup 1) or in those with 2-h PG values of <200 mg/dL (subgroup 2), MetS was positively associated with carotid plaque after adjustment for only age (Model 2), although the relationship was only borderline positive in women. In men, the association between MetS and carotid intima-media thickening was also statistically significantly positive after adjustment for only age. These associations lost statistical significance after adjustment for TC, smoking status, and components of MetS (Model 4), suggesting that these associations may not be independent of these factors. Attention should be given to the fact that after excluding individuals with hypertension from the analysis, the association between MetS and carotid plaque or carotid intima-media thickening was no longer statistically significant even after adjustment for only age (subgroup 3), which is in agreement with our previous finding [8].

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Several previous cross-sectional and longitudinal studies have investigated whether MetS increases the risk for atherosclerotic diseases in subjects without apparent impairment in glucose metabolism. A prospective population-based study of Finnish men showed that MetS was associated with higher mortality from coronary heart disease in men without impaired fasting glycemia [16]. Wilson et al. reported that MetS was associated with increased risk for cardiovascular disease in those without diabetes [17]. Leoncini et al. reported that MetS was associated with carotid atherosclerosis in non-diabetic hypertensive individuals who attended an outpatient clinic in Italy [18]. Kawamoto et al. analyzed Japanese inpatients and found that MetS increased the risk for carotid intima-media thickening in non-diabetic subjects [19]. Tzou et al. reported that the presence of MetS increased the composite of carotid intima-media thickness of ≥75th percentile of enrolled subjects in non-diabetic young adults [20]. These results support the notion that the presence of MetS will increase the risk for carotid atherosclerosis even in non-diabetic populations; however, caution should be paid in interpreting these results, as these results were not always adjusted for each component of MetS. The present results showed that MetS was associated with carotid plaque and intima-media thickening in men in subgroups 1, and 2 after adjustment for age, TC, and smoking status, although statistically significance would be lost after further adjustment for MetS components.

We found that in the absence of hypertension (subgroup 3), the association between MetS and carotid plaque or intima-media thickening was no more statistically significant after adjustment for only age, or even when no adjustments were made. These data collectively suggested that the presence or absence of hypertension, but not an abnormality in glucose metabolism, is crucial to determine whether the presence of MetS would increase the risk for carotid atherosclerosis. A recent study showed that MetS significantly increased all-cause mortality in hypertensive community-based French individuals with a hazard ratio of 1.40 (95% CI 1.13–1.74), but not in non-hypertensive individuals, during a mean follow-up period of 4.7 years [21], which was consistent with the idea of major role played by hypertension.

This study has several limitations. First, due to the cross-sectional nature of the study, we cannot determine whether there is a causal or resultant relationship between the MetS and presence of atherosclerosis. Second, among 8048 individuals who were not taking anti-diabetic medication, we excluded 4144 individuals who did not undergo OGTT. The mean age of the 3904 individuals who underwent OGTT and those 4144 who did not were significantly different (55 ± 10 years versus 58 ± 10 years, respectively, $P < 0.001$); therefore, it could be said that there had been some selection bias, though, again, the type of health screening was not decided or recommended by the physicians.

In conclusion, we showed that MetS was associated with carotid plaque and carotid intima-media thickening in non-diabetic individuals; although, this relationship did not remain statistically significant after adjustment for MetS components. In non-diabetic non-hypertensive individuals, the association between MetS and carotid plaque or carotid intima-media thickening was not statistically significant when adjustment was made for only age or even when no adjustment were made. These data collectively indicate that presence or absence of hypertension, but not an abnormality in glucose metabolism, is crucial to determine the relationship between MetS and carotid atherosclerosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2008.10.022.

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Virus associated innate immunity in liver

Tatsuya Kanto

Departments of Gastroenterology, Hepatology and Department of Dendritic Cell Biology and Clinical Applications, Osaka University Graduate School of Medicine

TABLE OF CONTENTS

1. ABSTRACT

Dendritic cells (DCs) sense virus via toll-like receptors (TLR) or retinoic acid inducible gene-I (RIG-I) and evoke a cascade of immune reactions. In myeloid DC (MDC) from hepatitis C virus (HCV)-infected patients, the levels of TLR/RIG-I-mediated IFN-beta or TNF-alfa induction are lower than those in uninfected donors, suggesting that their signal transduction in MDC is impaired. Dendritic cells in HCV infection are unresponsive to interferon (IFN)-alfa, thus failing to enhance MHC class-I related chain A/B and subsequent NK cell activation. Alternatively, NK cells from the patients down-regulate DC in the presence of HLA-E-expressing hepatocytes by secreting IL-10 and TGF-beta1. Such functional alteration of NK cells in HCV infection is ascribed to the enhanced expression of NKG2A/CD94. Activated NKT cells from the patients produce higher levels of IL-13 but comparable IFN-gamma with those from controls, showing their bias to Th2-type. In pegylated IFN-alfa/ribavirin therapy for chronic hepatitis C, improved DC function is related with successful HCV eradication. In conclusion, cross-talks among DCs and innate lymphocytes are critical in shaping immune response against HCV, either spontaneously or therapeutically.

2. INTRODUCTION

Hepatitis C virus (HCV) is one of major causes of chronic liver disease worldwide. HCV is hepatotrophic, but not directly cytopathic and elicit progressive liver injuries resulting in end-stage liver disease unless effectively eradicated (1). Epidemiological studies have revealed that more than 80% of acutely HCV-infected patients fail to eradicate the virus and they subsequently develop chronic hepatitis (1). It has been proposed that the ability of infected hosts to mount vigorous and sustained cellular immune reactions to HCV is necessary for control in primary infection (2). Once HCV survives the initial interaction with the host immune system, it uses several means to nullify the selective immunological pressure during the later phases of infection. First, the virus alters its antigenic epitopes recognized by T cells and neutralizing antibodies to escape immune surveillance. Second, HCV also subverts immune functions in an antigen-specific manner, from innate to adaptive immunity (3).

Cumulative reports have shown that innate immune system dictates the direction and magnitude of subsequent adaptive immune response. It is generally accepted that HCV-specific $CD8^+$ T cells are responsible

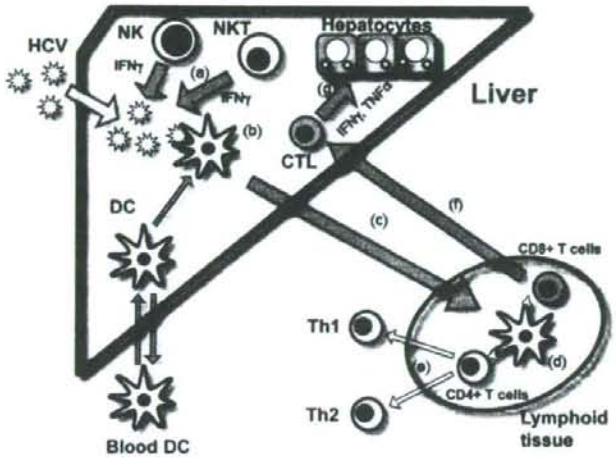

Figure 1. Key players in immune reactions in viral hepatitis. CTL, cytotoxic T lymphocyte; DC, dendritic cell; HCV, hepatitis C virus; NK, natural killer cell; Th, helper T cell. (a)- (g), see text.

for HCV elimination by inducing hepatocyte apoptosis (2). Innate immune cells, including NK cells and NKT cells, may contribute to HCV eradication after primary infection; however, their roles in chronically-infected state remain elusive. Since dendritic cells (DCs) orchestrate anti-HCV immune response by linking innate and adaptive arms of immune system (4), functional impairment of DC leads to failure of NK cells, NKT cells, $CD4^+$ and $CD8^+$ T cells. Infiltration of disabled $CD8^+$ T cells to the infected liver may result in weak liver inflammation that is not sufficient for HCV eradication (5).

In this paper, we discuss the current understandings of the roles of innate immunity in the pathogenesis of HCV infection as well as efficacy of anti-HCV therapy, especially focused on interferons (IFN), DCs, NK cells and NKT cells.

3. KEY PLAYERS IN IMMUNE RESPONSES TO VIRAL HEPATITIS

After HCV infects the liver, viral replication continues and viral particles are continuously released into the circulation. The first lines of defense are provided by NK and NKT cells, of which populations are relatively increased in the liver compared to the periphery. These cells are activated in the liver, where expression of IFN-alfa and IFN-inducible genes are extremely high during the early phase of hepatitis virus infection (6). Activated NK and NKT cells secrete IFN-gamma, which inhibits replication of HCV through a non-cytolytic mechanism (Figure 1-a) (7).

Dendritic cells (DCs) or resident macrophages in the liver are capable of taking up viral antigens, and processing and presenting them to other immune cells (Figure 1-b) (4). Since DCs express distinct sets of toll-like receptors (TLRs) (8), it is likely that some viral components stimulate DCs through cytosolic ligation of TLRs. DCs develop a mature phenotype and migrate to lymphoid tissues (Figure 1-c), where they stimulate effectors, including T cells and B cells (Figure 1-d). Following the encounter of DCs with other cells, DCs secrete various cytokines (IL-12, TNF-alfa, IFN-alfa and IL-10) instructing or regulating the functions of the adjacent cells (4). In addition to these cytokines, DCs express various co-stimulatory molecules and ligands to enhance or limit the functions of immune and infected cells. The existence of functionally and ontogenetically distinct DC subsets has been reported; i.e., myeloid DC (MDC) and plasmacytoid DC (PDC) (9). MDC predominantly produce IL-12 or TNF-alfa following pro-inflammatory stimuli, while PDC release a considerable amount of IFN-alfa upon virus infection depending on the immune stimulus; both cytokines in actuality can be made by both cells. Helper T cells have an immunoregulatory function mediated by the secretion of cytokines that support either cytotoxic T lymphocyte (CTLs) generation (Th1 with secretion of IL-2, IFN-gamma and TNF-alfa) or B cell function and antibody production (Th2 with secretion of IL-4, IL-5, IL-10 and IL-13) (Figure 1-e). DC ontogeny and DC-derived cytokines are crucially associated with the polarization of helper T cell subsets.

It is generally accepted that adaptive immunity performs a critical role during the clinical courses of hepatitis. The involvement of antigen-specific $CD4^+$ T cells in HCV eradication has been well described during both acute or chronic infection (10). However, there is little evidence that $CD4^+$ T cells mediate direct liver cell injury in HCV infection. Thus, it is likely that $CD4^+$ T cells play a critical role in facilitating other antiviral immune

mechanisms, such as enhancing $CD8^+$ effector function. The antigen-primed CTLs recruit to the liver (Figure 1-f) and constitute the critical element in the eradication of virus-infected cells (Figure 1-g).

4. INNATE IMMUNITY IN HCV INFECTION

4.1. Toll-like receptors and retinoic acid inducible gene-I as sensors for virus infection

Gene expression analyses in HCV-infected liver revealed that HCV triggers expression of type I IFN and IFN-induced genes during primary infection regardless of the outcomes (6). However, the HCV viral load does not decrease in the early phase, suggesting that HCV impedes the execution of anti-viral machineries. Several HCV-derived proteins are involved in the suppression on the signaling pathways inducing anti-viral proteins, such as interferon regulatory factor (IRF)-3 (11), NF-kappa B and RNA-dependent protein kinases (PKR) (12). Mammalian toll-like receptors (TLRs) sense some pathogen-associated molecular patterns embedded in virus components and then induce inflammatory cytokines or type-I IFNs, resulting in the augmentation of anti-virus immune reactions (8). Retinoic acid inducible gene-I (RIG-I) is a cytosolic molecule that senses dsRNA of virus replicative intermediate, which subsequently activates IRF-3 and NF-kappa B pathways (13). By using HCV subgenomic replicon system, it has been demonstrated that HCV NS3/4A proteins influences on the functions of adaptor molecules mediating TLR-dependent and RIG-I-dependent pathways, resulting in an impairment of the induction of IFN-beta as well as subsequent interferon-stimulated genes (14, 15). However, it is yet to be proven whether the results obtained from HCV replicon are applicable or not for HCV-infected individuals.

Large-scale cohort study on US veterans revealed that the prevalence of various infectious diseases, including virus, bacteria and parasites, in HCV-infected individuals is significantly higher than those in uninfected controls (16). These observations suggest that first-line defense against pathogens, of which system is initiated by TLR/RIG-I stimulation, is functionally impaired in HCV infection. To investigate the roles of TLR/RIG-I in HCV infection, we compared their expressions and the functions in MDC and PDC between the patients and donors. In MDC from HCV-infected patients, TLR2, TLR4 and RIG-I expression were significantly higher than those in healthy counterparts. Of particular interest, regardless of the higher expressions, specific agonists for these sensors stimulated patients MDC to induce lesser amount of IFN-beta and TNF-alfa compared to donor MDC (unpublished data). These results show that the signal transduction via these receptors is strongly impeded in HCV infection. Inconsistent with the findings of MDC, we previously reported that TLR2 expression on monocyte-derived DCs (MoDCs) in chronic hepatitis C is lower than those in healthy donors (17). Since MoDC is an in vitro-generated DC mimic, the opposite results of TLR2 in HCV infection might be explained by impaired ability of MoDC to mature in response to cytokines, as reported elsewhere (18). Further investigation is needed to clarify which TLR or RIG-I is predominantly utilized by HCV to evoke immune reactions.

4.2. Blood DC subsets

Impaired antigen presentation by DC might be involved in the failure of the maintenance of sustained HCV-specific T cell response. Monocyte-derived DCs (MoDCs) generated from hepatitis C patients have an impaired ability to stimulate allogeneic $CD4^+$ T cells (19, 20). Functional impairment of DC diminished when HCV had been eradicated from patients, revealing the evidence of HCV-induced DC disability (19). In addition to in vitro-generated DCs, the alterations in number and function of circulating blood DC have been reported in HCV infection (21, 22).

Direct HCV infection of DCs might be one of the plausible mechanisms of DC dysfunction in chronic hepatitis C. The HCV genome has been reported to be isolated from MoDCs or blood DCs (19). However, these results need to be interpreted carefully, since contamination with free virus in blood cannot be ruled out when amplifying PCR techniques are used. To exclude this possibility, HCV pseudovirus has been developed to investigate the cell tropisms of HCV as well as to determine putative HCV entry receptors to cells. By using this, MDC, but not PDC, displayed susceptibility to HCV pseudovirus possessing chimeric HCV E1/E2 proteins (23).

Several criticisms have been raised recently about DC dysfunction in the setting of chronic HCV infection (24), failing to demonstrate any DC defects which may have to do with differences in the populations studied. Cohort studies on chimpanzees following HCV infection showed that functional impairment of DCs was observed in some cases but was not a prerequisite of persistent infection (25). Further study needs to be done to clarify whether DCs are indeed disabled in the setting of human chronic hepatitis C and furthermore whether this contributes to the development of HCV persistence or it is simply a consequence of active HCV infection.

4.3. Natural killer cells

Natural killer cells express various functional receptors; the one group that transduces inhibitory signals (Killer Inhibitory Receptors/KIRs, CD94, NKG2A) and the other does activating signals (NKG2D). The function of NK cells is dynamically regulated in vivo by the balance between expressions of counteracting receptors and their association with relevant ligands (26). First, we compared the expressions of NK cell receptor between HCV-infected patients and healthy donors. As for inhibitory receptors, KIR expressions are not different between the groups; however, CD94 and NKG2A expressions are higher in patients than controls (27). In contrast, activating receptor NKG2D expression is comparable between the groups (Figure 2). It is yet to be determined how the expression of NK cell receptor is regulated. In our hands, HCV pseudovirus did not enter purified NK cells, suggesting that NK cells are not susceptible to direct HCV infection (unpublished data). Thus, some soluble factors and/or direct binding of HCV particles to NK cells might be the cause of NK receptor dysregulation.

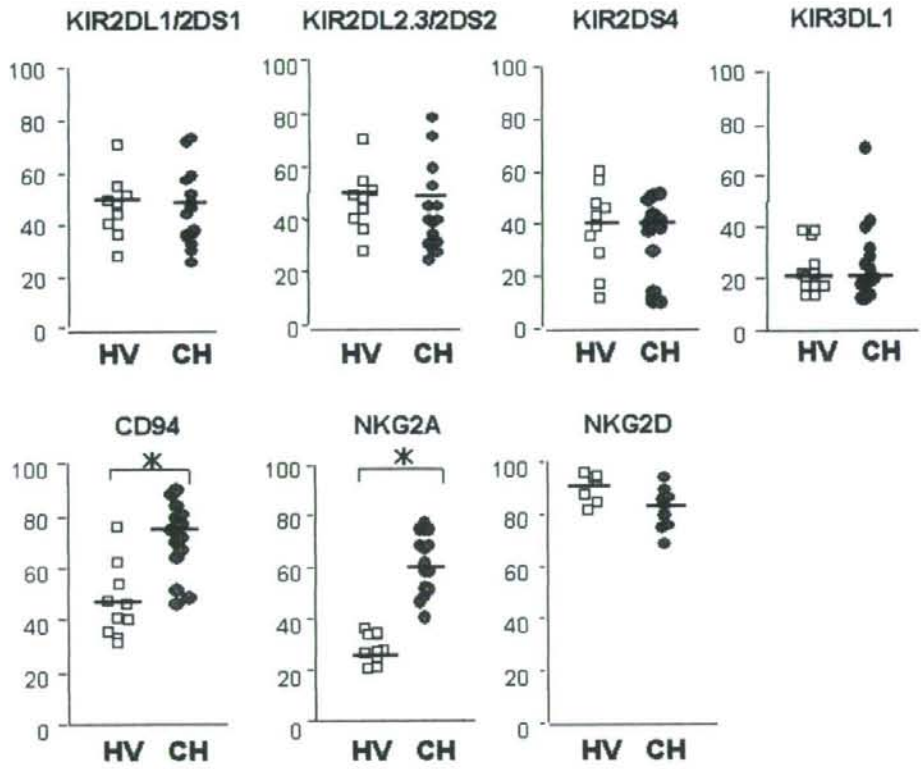

Figure 2. The expressions of NK receptors on NK cells from chronic hepatitis C patients and healthy subjects. The percentages of NK cell that express various NK receptors were determined by flow cytometry. HV, healthy volunteers; CH, chronic hepatitis C patients. Horizontal bars represent the median. $^{*}P < 0.05$ by Mann-Whitney U test.

DCs play a decisive role in shaping innate immunity by interacting with NK cells. DCs have two means to stimulate NK cells via the production of cytokines (IL-12, IL-18 or IFN-alfa) and through the expression of NK-activating ligands. In response to IFN-alfa, DCs are able to express MHC class-I related chain A/B (MICA/B) and activate NK cells following ligation of the NK receptor, NKG2D (28). Interestingly, DCs from HCV-infected patients are unresponsive to exogenous IFN-alfa to enhance MICA/B expression and fail to activate NK cells (28). It is tempting to speculate that the impairment of DCs in NK cell activation is responsible for the failure of HCV control in the early phase of primary HCV infection, where HCV continues to replicate in spite of high-level IFN-alfa expression in the liver. Alternatively, NK cells from HCV-infected patients down-regulate DC functions in the presence of hepatocytes by secreting suppressive cytokines, IL-10 and TGF-beta1 (27). Such functional alteration of NK cells in HCV infection was ascribed to the enhanced expression of inhibitory receptor NKG2A/CD94 compared to the healthy counterparts (27). Further study is necessary to determine if the NK-mediated DC suppression is instrumental or not in acute HCV infection.

4.4. Natural killer T cells

Natural killer T (NKT) cells are a unique lymphocyte subset co-expressing T-cell receptor (TCR) and NK cell markers (29). The NKT cell population is highly heterogeneous according to the differences in types and tissue distribution; invariant (or classical) NKT (iNKT) cells express an invariant TCR, composed of Valfa24-JalfaQ preferentially paired with Vbeta11 in humans (29), whereas non-invariant NKT cells express diverse TCR. Invariant NKT cells recognize glycolipid antigens presented on CD1d expressed by DCs (29). Although endogenous ligands of iNKT cells are little known, alfa-galactosyl-ceramide (alfaGalCer) has been used as a surrogate for natural ligands. In contrast, non-invariant NKT cells are activated by CD1d-dependent manner but are not reactive to alfaGalCer. Baron *et al.* reported that, in hepatitis B virus-transgenic mice, non-invariant NKT cells are critically involved in acute liver injury (30). As for a human counterpart, Exley *et al.* observed that CD1d

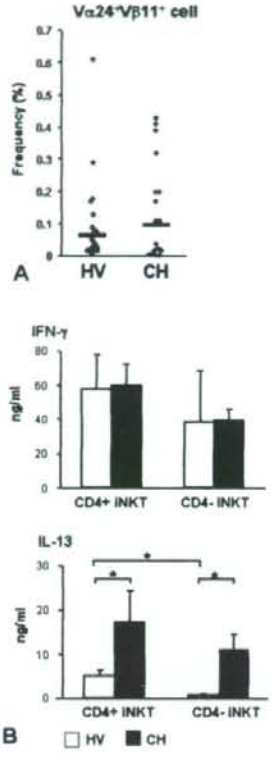

Figure 3. Frequency and cytokine production of invariant NKT cell subsets in healthy subjects and chronic hepatitis C patients. (A) The frequencies of total invariant NKT (iNKT) cells (Valfa24$^+$Vbeta11$^+$ cells) in PBMCs were determined by flow cytometry. HV, CH; See Fig 2. Horizontal bars represent the median. (B) Invariant NKT (iNKT) cells were expanded by culture with alfaGalCer-pulsed autologous monocyte-derived DCs (MoDCs) and CD4$^+$ and CD4$^-$ iNKT cells were collected by subsequent cell sorting. The activated iNKT cells were stimulated with alfaGalCer-pulsed allogeneic MoDCs for 24 h and the supernatants were collected for cytokine ELISA. The bars represent mean $\pm$ SE of 5 different subjects. HV, CH; See Figure 2. $^*P < 0.05$ by Mann-Whitney U test.

restricted non-invariant NKT cells infiltrate in HCV-infected liver, where they presumably exert their promoting role in liver inflammation (31). Hepatic inflammatory cells or biliary cells up-regulate CD1d which subsequently supports NKT cell activation (32). In addition, hepatic stellate cells are capable of activating NKT cells via surface CD1d and secretion of IL-15 (33).

Although iNKT cells comprise a small portion of hemotopoietic cells, they regulate various immune responses by secreting Th1 as well as Th2 cytokines in clinical settings. It has been demonstrated that phenotypic as well as functional subsets exist for iNKT cells, which are CD4$^+$, CD4$^-$CD8$^-$ double negative (DN) and CD8$^+$ ones. The CD4$^+$ and DN iNKT cells produce both Th1 (IFN-gamma) and Th2 cytokines (IL-4, IL-5, IL-13). The CD4$^+$ iNKT cells secrete more Th2 cytokines than DN, while CD8$^+$ subsets predominantly secrete Th1 cytokines (34). For chronic HCV infection, some controversial reports have been published about the frequency of iNKT cells (35, 36), however, their functional roles in HCV-infected patients are largely unknown. We thus compared the frequency and the cytokine producing capacity of iNKT cells in peripheral blood between chronic hepatitis C patients and healthy individuals. Furthermore, to analyze the functions of activated iNKT cells, we expanded iNKT cells by the stimulation with alfaGalCer-loaded DCs. We demonstrate that the number and functions of iNKT cells from HCV-infected patients are comparable with those from healthy subjects at the steady state (Figure 3A) (37). By contrast, activated iNKT cells from patients released more Th2 cytokines, most significantly IL-13, than those from the controls (Figure 3B) (37). Recently, other groups have reported that IL-4 and IL-13 from fresh iNKT cells were increased in liver cirrhosis caused by HBV or HCV, implying that these cells are pro-fibrogenic to the liver (38). If this is the case, our findings suggest that iNKT cells in chronic HCV infection are pro-fibrogenic per se even in the pre-cirrhotic stage. The reason why iNKT cells in HCV infection are Th2-biased needs to be further investigated.

5. ADAPTIVE IMMUNITY IN HCV INFECTION

Many reports have been published on the importance of CD4$^+$ T cell response in the clearance and control of HCV. In chronic hepatitis C patients, HCV-specific CD4$^+$ T cells were functionally impaired and their activity was not sustained (39), which was in clear contrast with resolved cases. Inoculation studies of infectious HCV to recovered chimpanzees demonstrated that CD4$^+$ T cell help was indispensable for the development of effective CD8$^+$ T cell response to protect from HCV persistence (40).

With regard to HCV-specific CD8$^+$ T cells observed during the chronic stages of disease, conflicting results have been reported for their roles in HCV replication and liver inflammation. Several investigators have shown that the HCV-specific CTL response is inversely correlated with viral load, suggesting its inhibitory capacity on HCV replication (41). However, others did not find a significant relationship between these parameters (42). HCV-specific CD8$^+$ T cells in chronic hepatitis C patients possess lesser capacity to proliferate and produce less IFN-gamma in response to HCV antigens. Since CD8$^+$ T cells are reported to be involved in HCV-induced liver inflammation, inefficient CD8$^+$ T cells may evoke only milder hepatocyte injury, which level is not sufficient for HCV eradication (5).

Several plausible mechanisms have been proposed for T cell functional failure observed in chronic HCV infection (3): 1) HCV escape mutation, 2) primary T

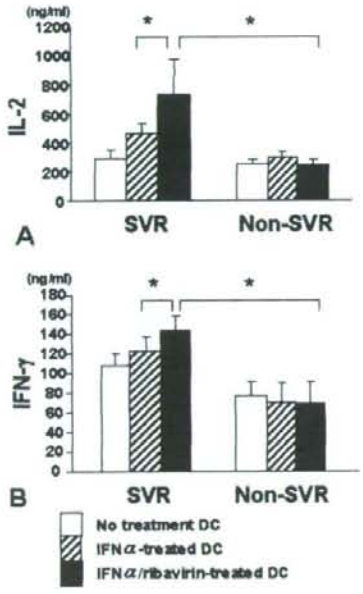

Figure 4. Improvement of Th1-inducing ability of dendritic cells by IFN-alfa and ribavirin from chronic hepatitis C patients with sustained virological response in combination therapy. Monocyte-derived dendritic cells (DC) were generated with GM-CSF and IL-4 in the presence or absence of IFN-alfa and ribavirin and were cultured with allogeneic naïve $CD4^+$ T cells for 6 days. On day 4 of the culture, half of the supernatants were collected for the assessment of IL-2. After 6 days, the cultured cells were stimulated with phorbol myrisate acetate and ionomycin for 24 hours. The results of IFN-gamma (A) and IL-2 (B) determined by ELISA were compared among them in the SVR and non-SVR group. The results are expressed as mean ± SE from five SVR and nine non-SVR patients. SVR, sustained virological responder in 24 weeks of IFN-α and ribavirin therapy. $^*P < 0.05$ by Mann-Whitney U-test.

cell failure or T cell exhaustion, 3) impaired antigen presentation, 4) suppression by HCV proteins, 5) impaired T cell maturation, 6) suppression by regulatory T cells and 7) tolerogenic environment in the liver.

6. IMMUNE RESPONSE DURING ANTI-VIRAL THERAPY

Anti-viral agents, pegylated (PEG) IFN-alfa and ribavirin, have been widely used for the treatment of chronic HCV infection in order to prevent the development to liver cirrhosis and hepatocellular caricinoma (1). In addition to providing direct inhibition of viral replication, these agents modulate antiviral immune responses, which greatly contribute to the successful therapeutic response. Earlier studies reported that HCV-specific $CD8^+$ T cell response, as examined by CTL precursor frequency, was not enhanced after IFN-alfa monotherapy (43). Furthermore, analyses of MHC class-I tetramer-positive cells in patients who underwent IFN-alfa and ribavirin therapy revealed that $CD8^+$ T cells did not increase following treatment and they were not associated with outcome (44). Combination therapy of IFN-alfa and ribavirin increases antigen-specific $CD4^+$ T cell proliferation and IFN-gamma production by $CD4^+$ T cells (45, 46). The "vigor" of the $CD4^+$ T cell response to HCV eradication is reported to be variable, something which is considered quite controversial (44).

Currently, no data is available for the involvement of innate immunity in the efficacy of IFN-alfa-based anti-HCV therapy. We thus examined whether IFN-alfa and ribavirin give a positive impact on DC capacity to induce $CD4^+$ T cell (Th1) response. By using in vitro culture system, monocyte-derived DC from chronic hepatitis C patients were impaired in the ability to drive Th1 in response to IFN-alfa. When we compared such DC capacity between patients who cleared HCV (sustained virological responders, SVR) by IFN-alfa/ribavirin therapy and those who failed to do so, impaired DC function was restored in response to IFN-alfa/ribavirin in SVR patients but not in non-SVR ones (Figure 4) (47). These results imply that DC responsiveness to anti-viral agents is restored in patients who potentially gain favorable outcomes in IFN-alfa/ribavirin therapy.

Next, we aimed to elucidate if the frequency or function of DC and innate lymphocytes is related to the outcome of pegylated IFN-alfa and ribavirin therapy. In comparison with SVR patients, non-SVR ones and transient responders (TR) showed a decline of PDC frequency from weeks 1-12 and impaired DC function at the end of treatment (Figure 5A) (48). The frequency of NK cells, as defined as $CD3^-CD56^+$ cells, in SVR patients was lower than those in TR ones (Figure 5B). In contrast, the frequency of invariant NKT cells ($Valfa24^+Vbeta11^+$ cells) did not differ between the groups in the course of the treatment (data not shown). These results show that restoration of DC function is critically involved in favorable response in pegylated IFN-alfa/ribavirin therapy. In other words, DC system could be a target of therapeutic immune modulation.

The questions remain unsolved are if impaired immune system in chronic HCV infection is restored or not by the successful HCV eradication after anti-viral therapy. Controversial results have been reported about the durability of treatment-induced recovery in HCV-specific immune response (49, 50), which seems to be clearly distinct from that observed in spontaneous HCV resolvers.

7. PERSPECTIVE

Protease inhibitors against HCV NS3/4A are now ready to use in clinics (51). Since they possess potent ability to suppress HCV replication, they are quite promising as an alternative approach for non-responders in PEG-IFNalfa/ribavirin therapy. In addition to that, it is anticipated that protease inhibitors are able to restore innate

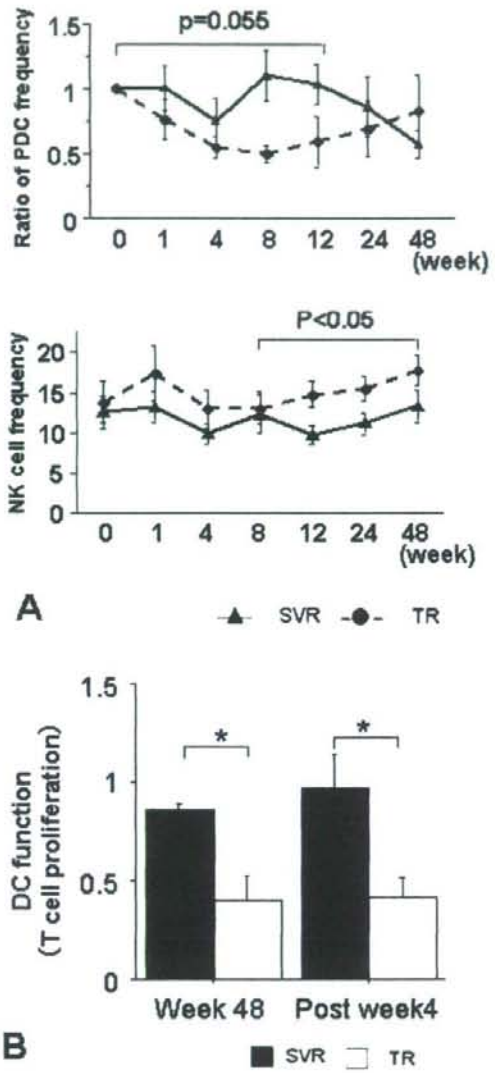

Figure 5. Early phase decline pf plasmacytoid dendritic cell frequency and sustained impairment of dendritic cell ability are related to transient response in 48-week pegylated IFN-alfa and ribavirin therapy. Frequencies and their ratios of plasmacytoid dendritic cells (PDC) and NK cells in the patients during the pegylated IFN-alfa and ribavirin therapy were determined by flow cytometric analysis. PDC were defined as Lineage-negative, HLA-DR$^+$, CD11c$^-$ and CD123^{high} cells and NK cells were as CD3-negative and CD56$^+$ cells, respectively. The results are expressed as mean $\pm$ SE. *P < 0.05 by ANOVA. At the end of treatment (Week 48) and at Week 4 after the completion of therapy, monocyte-derived DC were generated from the patients or healthy donors and their allostimulatory capacity was evaluated by mixed lymphocyte reaction (MLR). The MLR ratio between patients and controls was determined from the counts per minute of ^{3}H-thymidine incorporated into CD4$^+$ T cells at T cell/DC ratio of 10/1. The results are expressed as the mean $\pm$ SE of 11 SVR and 11 transient responders. SVR and TR, sustained virological responders and transient responders in 48 weeks of pegylated IFN-alfa and ribavirin therapy.*P < 0.05 by Mann-Whitney U test.

immunity by disarming NS3/4A-mediated suppression on TLR/RIG-I-dependent or -independent pathways. Therefore, extensive immunological studies on the patients treated with protease inhibitors are needed to elucidate if the therapeutic modulation of innate immunity could shape HCV-specific adaptive immunity or not. The next steps in evolving innovative approaches to establish HCV-specific immunotherapy are to determine the means to, direct the magnitude, breadth, quality and duration of antigen-specific immune responses in a desired way. Active modulation of innate immunity may be one of the strategies to gain access to the goal.

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Abbreviations: CTL, cytotoxic T lymphocytes; DC, dendritic cells; HCV, hepatitis C virus; IFN, interferon; MICA, MHC class-I related chain; MDC, myeloid dendritic cells; Mo-DC, monocyte-derived dendritic cells; NK, natural killer; PDC, plasmacytoid dendritic cells; RIG-I, retinoic acid inducible gene-I, SVR, sustained virological responders; TLR, Toll-like receptors; TCR, T cell receptor; TR, transient responders

Key Words: Dendritic cells, NK cells, NKT cells, Toll-like receptor, RIG-I, pegylated interferon-alfa, ribavirin, Review

Send correspondence to: Tatsuya Kanto, Department of Gastroenterology and Hepatology and Department of Dendritic cell Biology and Clinical Applications, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871 Japan, Tel: 81-6-6879-3446, Fax: 81-6-6879-3448, E-mail: kantot@gh.med.osaka-u.ac.jp

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A Single Amino Acid of Toll-like Receptor 4 That Is Pivotal for Its Signal Transduction and Subcellular Localization*

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Shintaro Yanagimoto[‡§¶], Keita Tatsuno[§], Shu Okugawa[§], Takatoshi Kitazawa[§], Kunihisa Tsukada[§], Kazuhiko Koike[§], Tatsuhiko Kodama[¶], Satoshi Kimura[‖], Yoshikazu Shibasaki[‡1], and Yasuo Ota[1,2]**

From the [‡]Center for Structuring Life Sciences, Graduate school of Arts and Sciences, University of Tokyo, Meguro-ku, Tokyo 153-8903, the [§]Department of Infectious Diseases, Graduate School of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113-8655, the [¶]Laboratory for Systems Biology and Medicine, Research Center for Advanced Science and Technology, University of Tokyo, Meguro-ku, Tokyo 153-8904, the [‖]Tokyo Teishin Hospital, Fujimi, Chiyoda-ku, Tokyo 102-8798, and the [**]Department of Medicine, Teikyo University School of Medicine, 2-11-1, Kaga, Itabashi-ku, Tokyo 173-8605, Japan

Toll-like receptor 4 (TLR4) is essential for recognizing a Gram-negative bacterial component, lipopolysaccharide (LPS). A single amino acid mutation at position 712 of murine TLR4 leads to hyporesponsiveness to LPS. In this study we determined that an amino acid, a leucine at position 815 of human TLR4, is also pivotal for LPS responsiveness and subcellular distribution. By replacing the leucine with alanine, the mutant TLR4 lost responsiveness to LPS and did not localize on the plasma membrane. In addition, it does not coprecipitate with myeloid differentiation-2, an accessory protein that is necessary for TLR4 to recognize LPS. These results suggest that the leucine at position 815 is required for the normal maturation of TLR4 and for formation of the TLR4·MD-2 complex.

Toll-like receptors (TLRs)[3] play essential roles in both innate and adaptive immunity (1). Thirteen members of the TLR family have been identified in mammals. TLRs have leucine-rich-repeats in their extracellular domains and a Toll/Interleukin-1 receptor (TIR) in their cytoplasmic domains, the latter of which mainly mediates intracellular signaling. Signaling pathways of TLRs, except for TLR3, depend on an adapter protein, MyD88 (myeloid differentiation factor 88), which interacts with the TIR domain of TLRs. This pathway leads to the activation of the transcription factor NF-κB and production of cytokines such as tumor necrosis factor-α and interleukin-6. Another important signaling pathway mediated by TLR3 and TLR4 that exploits the TIR domain is the MyD88-independent pathway. This pathway involves different adapter proteins, such as the TIR domain-containing adaptor inducing interferon-β (TRIF) and TRIF-related adaptor molecule (2–4), and is essential for production of type I interferon through activation of interferon regulatory factor-3.

TLRs recognize as ligands several microbial pathogen-associated molecular patterns. One such pathogen-associated molecular pattern is lipopolysaccharide (LPS), which is recognized by TLR4. LPS triggers severe immunologic reactions by the host in Gram-negative bacterial infections and has drawn attention in many clinical situations. TLR4 is the first mammalian TLR to be discovered in the context of immunology. TLR4 was identified in the search for the genes responsible for LPS hyporesponsiveness (5, 6). The defect was found to stem from a single amino acid mutation, replacement of proline with histidine at position 712, in the cytoplasmic tail of murine TLR4. The study led to the discovery of the importance of TLR4 in innate immunity.

A variety of cells are activated by LPS stimulation through TLR4. TLR4 forms a receptor complex with an accessory protein, myeloid differentiation-2 (MD-2). MD-2 first associates with TLR4 in the endoplasmic reticulum (ER) and *cis*-Golgi, and both proteins move together to the plasma membrane (7, 8). Upon recognition of LPS, the TLR4·MD-2 complex receives LPS on the cell surface and initiates intracellular signaling. The expression of TLR4 in the absence of MD-2 does not confer full responsiveness to LPS stimuli in experimental cell lines (9). An analysis of MD-2 knockout mice revealed that MD-2 is important not only for LPS sensing but also for cellular distribution of TLR4.

In this study we hypothesized that the cytoplasmic tail of TLR4 contains regions that control both localization and signaling. Using truncation and mutation analysis, and paying particular attention to the TIR domain, we identified a single amino acid that is pivotal for both TLR4 signaling and subcellular distribution. The site we found was on the C-terminal portion of the TIR domain for which no specific function has been yet determined.

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[1] Both authors contributed equally to this work.

[2] To whom correspondence should be addressed. Tel.: 81-3-3964-1211 (ext. 1756); Fax: 81-3-3579-6310; E-mail: yasuota-tky@umin.ac.jp.

[3] The abbreviations used are: TLR, Toll-like receptor; TIR, Toll/Interleukin-1 receptor; TRIF, TIR domain-containing adaptor inducing interferon-β; LPS, lipopolysaccharide; MD-2, myeloid differentiation-2; ER, endoplasmic reticulum; GFP, green fluorescent protein; EGFP, enhanced GFP; RLA, relative luciferase activity; Sulfo-NHS-SS-Biotin, sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate.

EXPERIMENTAL PROCEDURES

Reagents and Other Materials—Lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 was purchased from Sigma-Aldrich and applied without repurification. FLAG- and hexa-histidine (His_6)-tagged human TLR4 expression plasmid (pEFBOS/humanTLR4flaghis) and FLAG- and His_6-tagged human MD-2 expression plasmid (pEFBOS/humanMD-2flaghis) were generous gifts from Dr. Kensuke Miyake (Institute of Medical Science, University of Tokyo, Japan). Human CD14 cDNA plasmid (pCMV6-XL5/humanCD14) was purchased from OriGene (Rockville, MD). Fluorescent protein expression vector pEGFP-N3 was purchased from Clontech (Mountain View, CA). Anti-TLR4 monoclonal antibody (clone HTA125) was purchased from Abcam (Cambridge, MA). Anti-FLAG monoclonal antibody (clone M2) was purchased from Sigma-Aldrich. Anti-*A.v.* (GFP) monoclonal and polyclonal antibodies were purchased from Clontech. Control immunoglobulins for immunoprecipitation were purchased from BD Biosciences (San Jose, CA). Horseradish peroxidase-labeled anti-immunoglobulins antibodies were purchased from Dako (Glostrup, Denmark). BlockAce (DS Pharma Biomedical, Osaka, Japan) solution was used as blocking buffer for Western blotting.

Cell Culture—Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich) containing 10% heat-inactivated fetal bovine serum supplemented with penicillin-streptomycin solution (Invitrogen). FuGENE 6 transfection reagent (Roche Applied Science) was used for transient cotransfection according to the manufacturer's instructions. Culture dishes or plates were prepared to 70% confluence prior to transfection. Cells were used for experiments 36 h later. The transfection conditions were optimized for microscopic observation of the expressed fluorescent protein and were kept unchanged in other experiments.

Expression Vector Subcloning and Mutagenesis—Wild-type TLR4 cDNA was excised from pEFBOS/humanTLR4flaghis and subcloned into pEGFP-N3 so that when expressed enhanced green fluorescent protein (EGFP) would be fused at the C terminus of TLR4 (pEGFP-N3/humanTLR4). All mutations were introduced into pEFBOS/humanTLR4flaghis and pEGFP-N3/humanTLR4 using the QuikChange site-Directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions and were confirmed by sequencing. For the truncation analysis, two identical unique restriction sites were prepared in the TLR4-coding region of pEFBOS/humanTLR4 using a QuikChange kit, and the DNA fragment to be removed, which was a part of the C terminus of TLR4, was excised enzymatically. After agarose gel purification, the linear double-stranded DNA was ligated to re-form a circular plasmid. Restriction sites were designed so as not to cause a frameshift between TLR4 and EGFP.

Confocal Laser Scanning Microscopy of Cells—Samples were fixed in 3% paraformaldehyde-phosphate-buffered saline at 37 °C for 10 min. Fluorescence images of fixed samples were recorded using a FluoView FV1000 Confocal Microscope (an inverted confocal laser scanning microscope, Olympus, Tokyo, Japan).

Immunoprecipitation—Transfected cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Triton X-100, 1 mM 1,4-dithiothreitol, and proteinase inhibitor mixture), sonicated, and centrifuged at 4 °C. Antibody was added to the supernatant, and the sample was rotated 1 h at 4 °C followed by the addition of protein G-Sepharose (GE Healthcare Life Sciences, Piscataway, NJ) and an additional 8-h incubation at 4 °C. Bound protein was washed three times in lysis buffer. Proteins were eluted by boiling in SDS sample buffer.

Biotinylation and Purification of Cell Surface Proteins—Prior to surface biotinylation, HEK 293T cells plated in a 100-mm dish were transiently transfected as described above. Surface biotinylation and subsequent purification of biotinylated proteins were performed using a Cell Surface Protein Biotinylation and Purification Kit (Pierce) following the manufacturer's instructions. Briefly, membrane-impermeable sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (Sulfo-NHS-SS-Biotin) was added to cell monolayers in the culture dishes and covalently bound to amines in proteins exposed on the cell surface. The affinity resin that binds to the biotin end of Sulfo-NHS-SS-Biotin was used to collect the biotinylated proteins. Reduction by 1,4-dithiothreitol causes cleavage of the disulfide bond in Sulfo-NHS-SS-Biotin, and the elute contains the biotinylated cell surface proteins. Each final sample obtained was considered to contain proteins from an equal amount of cells, because all culture plates were treated equally and grown to full confluence. All samples were sonicated and subjected to SDS-PAGE and Western blotting. The membrane to which protein was transferred was blocked in blocking buffer for 1 h. Then the membrane was incubated with a primary antibody, followed by incubation with horseradish peroxidase-labeled anti-immunoglobulins antibody. The protein bands were then visualized by using a chemiluminescence reagent, Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA), according to the manufacturer's instructions.

Cell Stimulation Assays—HEK293T cells were plated and transiently transfected for assays. Thirty-six hours after the transfection, LPS was added to fresh culture medium in each well of the culture plates at the stated concentration. The duration of LPS stimulation was 7 h.

Dual Luciferase Reporter Assays for NF-κB Activation—HEK293T cells were plated in 12-well culture plates (4×10^4 cells/well), and experimental cDNA plasmids were transiently transfected 36 h later using the FuGENE 6 transfection reagent with 0.5 μg of NF-κB reporter plasmid expressing firefly luciferase (pNF-κB-Luc, Stratagene) and 0.05 μg of constitutively active *Renilla* luciferase reporter plasmid (pRL-TK, Promega, Madison, WI) in addition to 0.5 μg each of TLR4-EGFP plasmid and MD-2 plasmid. Stimulation experiments were performed 36 h later. Firefly luciferase and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) and the Genelight55 luminometer (Microtech, Chiba, Japan). Relative luciferase activity (RLA) was obtained as the ratio of firefly luciferase activity to *Renilla* luciferase activity. Results are expressed as the ratio of RLA with LPS stimulation to RLA without LPS stimulation ([RLA LPS+]/[RLA LPS−]). This ratio should ideally approach 1 when no response to LPS stimulation is observed.

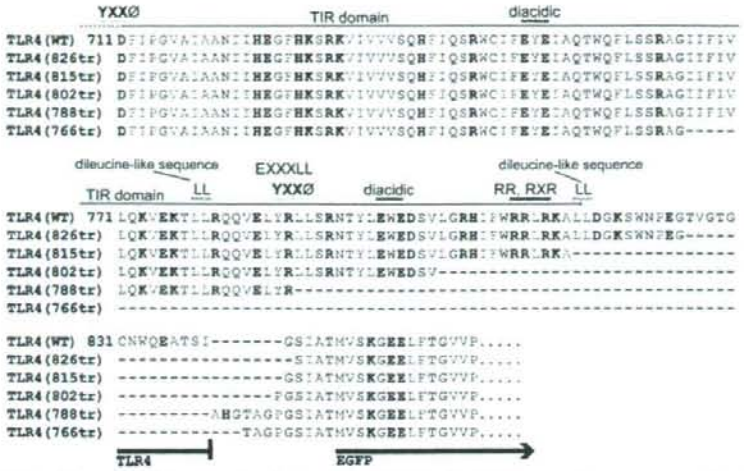

FIGURE 1. **Alignment of the cytoplasmic domains of EGFP fusion TLR4 truncation mutants used in this study.** TLR4 (766tr) signifies the mutant truncated at position 766. Others are named in the same manner. The amino acids are colored based on their physicochemical properties: *pink*, basic; *blue*, acidic; *green*, polar and neutral; and *orange*, hydrophobic. The *black overline* represents the TIR domain. *Colored overlines* indicate amino acid sequences identical to known sorting signal motifs except for two LLs, which are dileucine motif-like sequences in that they consist of solely two consecutive leucines without preceding aspartate or glutamate. *Capital letters* on the *line* signify the single-letter code for amino acids: *E*, glutamic acid; *L*, leucine; *R*, arginine; and *Y*, tyrosine. *X* signifies any amino acid, and Ø signifies an amino acid residue with a bulky hydrophobic side chain.

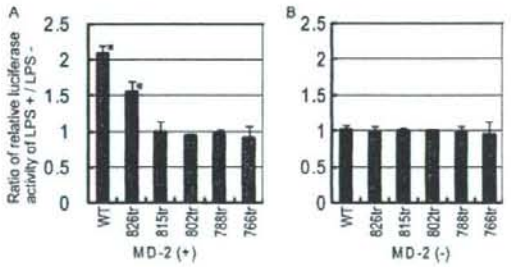

FIGURE 2. **LPS responsiveness measured by NF-κB luciferase assay.** HEK293T cells were transfected with plasmids containing the gene for wild-type TLR4 or a truncated human TLR4-EGFP fusion protein, in addition to a luciferase reporter and human MD-2 plasmid (*A*) or unmodified plasmids (control) (*B*). After 36 h, cells were stimulated with LPS (10 ng/ml) for 7 h, and luciferase reporter gene activity was measured. All results were expressed as the ratio of relative luciferase activity with LPS stimulation to that without stimulation. The data were from three independent experiments. *Small bars* indicate 95% confidence intervals of the mean (*p* values for * are: TLR4 (WT)-EGFP/MD-2 (+), $p = 0.002$; TLR4 (826tr)-EGFP/MD-2 (+), $p = 0.016$).

Statistical Analyses—All quantitative experiments were repeated three times, and each experiment was done in triplicate. The ratio of relative luciferase activity of LPS+ to LPS− was calculated as the index of the responsiveness to the stimuli as explained above. When positive response is observed, the ratio should significantly exceed one. The means of the ratio were represented in *bar graphs*. The 95% confidence interval of the mean of the ratio was calculated and indicated on each *bar* in the *graph*, and *p* values were calculated using Student's *t* distribution compared with the hypothetical mean, one.

RESULTS

Truncation Analysis of TLR 4—To identify amino acid sequences in the cytoplasmic tail of TLR4 that are involved in both signal transduction and subcellular distribution, first we generated five truncation mutants of TLR4 with a fluorescent protein (EGFP) at the C terminus of TLR4.

Although there are no known definite sorting signal motifs in the cytoplasmic tail of TLR4, some amino acid sequences are similar or identical to known general sorting signal motifs as shown in Fig. 1. *YXXØ*, a form of tyrosine-based sorting signal, and *EXXXLL*, a form of dileucine (LL)-based sorting signal, both control protein internalization, lysosomal targeting, and basolateral targeting (10), where "*X*" represents any amino acid, "Ø" stands for an amino acid residue with a bulky hydrophobic side chain, and other letters are single-letter abbreviations for the amino acids. "Diacidic" signals such as *DXE* mediate export from the ER (11). RR or *RXR* is another example of an ER export signal (12). Truncation sites were chosen so that some of these amino acid sequences were deleted in each mutant. Because the TIR domain, which is essential in TLR4 signaling and possibly subcellular localization (13), spans most of the cytoplasmic domain of TLR4, four out of five mutants have involvement in the TIR domain, which we hypothesized could result in impaired signal transduction and a change in subcellular distribution. Part of the cytoplasmic portion of the amino acid sequence of the truncation mutants is shown in Fig. 1. The five truncation mutant proteins lost their C-terminal tails at positions 826, 815, 802, 788, and 766, respectively, and were conjugated with EGFP *in vitro*. Actual truncation and ligation sites of all actual mutants were confirmed to have the designed DNA alignment by sequencing.

We utilized the luciferase reporter assay to assess NF-κB transcription activity as an indicator of TLR4 response to LPS stimuli. MD-2 is reported to be essential for this response (9). However, because it is not known whether MD-2 is necessary for transduction of the truncated TLR4 signal as well, we performed the assays with and without MD-2. The index of cell responsiveness to the stimulation was measured as the ratio between RLA with LPS stimulation and RLA without LPS stimulation. Only cells transfected with TLR4 (826tr)-EGFP in combination with MD-2 retained responsiveness to LPS stimulation. One exception was wild-type TLR4-EGFP (Fig. 2*A*). HEK293T cells transfected with TLR4 but without MD-2 did not respond to LPS stimuli regardless of the TLR4-EGFP genotype (Fig. 2*B*).

Next, we compared the localization of wild-type and truncated mutants of TLR4-EGFP in HEK293T by fluorescence microscopy (Fig. 3*A*). The wild-type TLR4 cotransfected with MD-2 was expressed on the plasma membrane and also in the

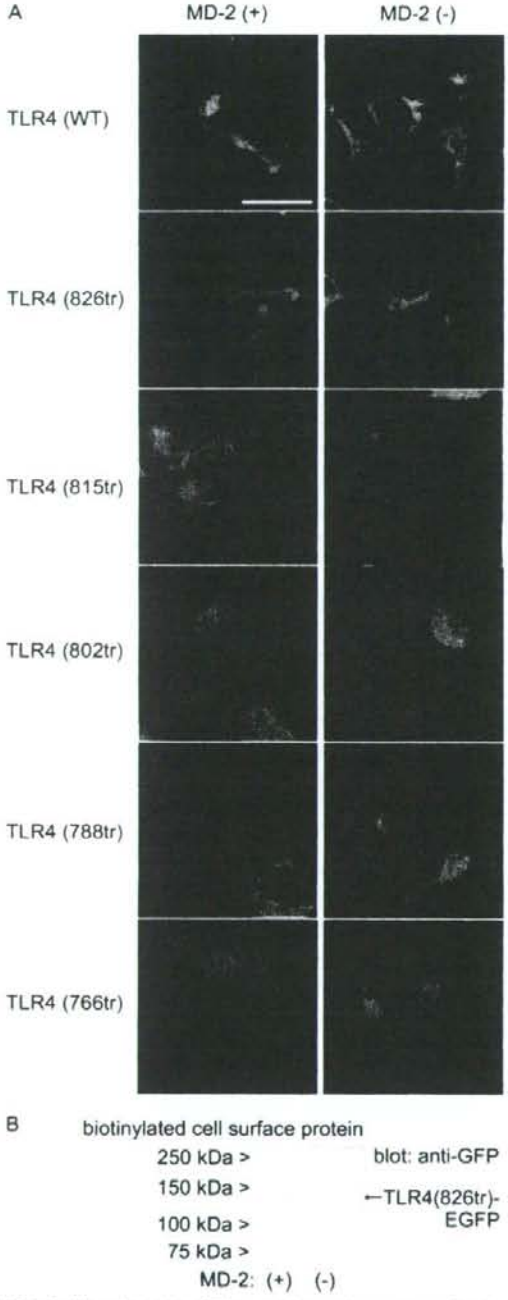

FIGURE 3. **Residues 815–826 of TLR4 contain a region necessary for plasma membrane localization.** *A*, cells were cultured on coverslips in 12-well plates and transfected as in Fig. 2. EGFP-tagged TLR4 was visualized by laser confocal microscopy. Fluorescence from EGFP was observed in *green*. Each genotype of TLR4-EGFP was cotransfected with a human MD-2 plasmid or empty vector. *Bar*, 20 μm. *B*, TLR4 (826tr)-EGFP with or without coexpression of MD-2 were tagged by biotinylation of the cell surface proteins and affinity-purified. TLR4 was visualized by immunoblotting using an anti-GFP monoclonal antibody. Samples from both combinations of DNAs were prepared from the same number of cells.

perinuclear area. These findings were consistent with observations by others (14, 15). TLR4 is reported to localize in the Golgi apparatus as well as on the plasma membrane. Our observation of TLR4-EGFP accumulation in the perinuclear area does not contradict the report that TLR4 partly localizes in the Golgi apparatus (14).

TLR4-EGFP truncation mutants, 815tr, 802tr, 788tr, and 766tr apparently did not localize at the plasma membrane. No particular fluorescence pattern that might be characteristic of localization to a specific intracellular compartment was observed. Only TLR4 (826tr)-EGFP, which has the shortest truncation, was expressed on the plasma membrane and in the perinuclear area, and the fluorescence pattern was similar to that of wild-type (Fig. 3*A*). No TLR4 genotypes, including wild-type TLR4-EGFP, clearly localized on the plasma membrane in the absence of MD-2 (Fig. 3*A*). MD-2 is reported to be necessary for localization of wild-type TLR4 at the plasma membrane (15), which is consistent with our observation. Intracellular distribution of mutant TLR4 varied depending on the genotype, but no particular cellular structure was identified as an alternative target site. Furthermore, we examined the plasma membrane expression of TLR4 (826tr)-EGFP by cell surface protein biotinylation. The expression level of TLR4 (826tr)-EGFP was markedly decreased without coexpression of MD-2 (Fig. 3*B*), which is compatible with the microscope observation.

Removal of the C-terminal segment of TLR4 at residue 826 does not qualitatively affect LPS responsiveness and subcellular distribution. However, when more residues, up to position 815, were removed, both signal transduction and plasma membrane localization were impaired. These results suggest that residues 815–826 of TLR4 contain at least one segment that is critical for those functions.

Amino Acid Sequence Replacement Analysis—To identify critical amino acid sequences in this region, we generated an amino acid replacement mutant of TLR4 instead of truncation mutants. As shown in Fig. 1, although it is not a canonical sequence, leucine-leucine at 815–816 partially fits a known sorting signal motif, a dileucine motif, (D/E)*XXX*L(L/I) or D*XX*LL, which plays an important role in internalization of plasma membrane protein or sorting from the *trans*-Golgi network (10). Thus, as has been done in a similar study (16), a mutant was generated in which alanines were substituted for both leucines at positions 815 and 816.

We measured the NF-κB activity of TLR4 (L815AL816A)-EGFP, the mutant in which both leucines were replaced with alanines, under LPS stimulation (Fig. 4*A*). This mutant protein did not respond to LPS stimuli. Microscopic observation revealed that TLR4 (L815AL816A)-EGFP was not expressed on the plasma membrane regardless of whether MD-2 was cotransfected (Fig. 4*B*). The phenotype of this doubly substituted mutant appeared to be the same as that of the truncation mutants. These results imply that the leucines in positions 815 and 816 play an important role in TLR4 plasma membrane localization.

Analysis of Single Amino Acid Substitution Mutants—As previously mentioned, the amino acid sequence leucine-leucine at positions 815 and 816 does not completely match the dileucine motif, *i.e.* it lacks a preceding acidic amino acid. Therefore it

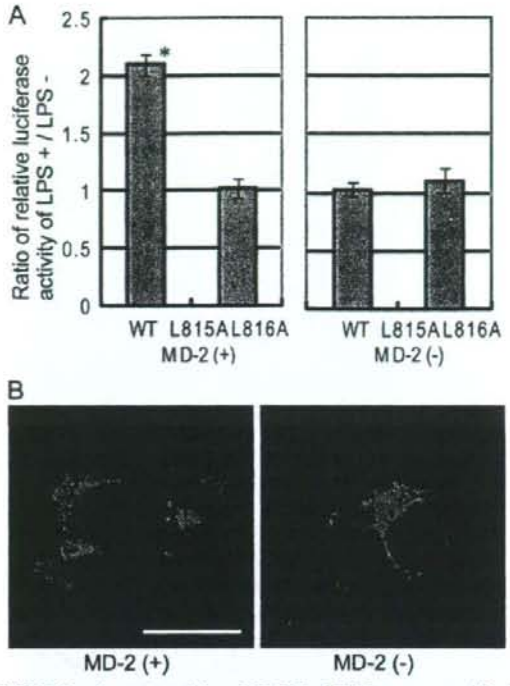

FIGURE 4. **Leucines at positions 815–816 of TLR4 are responsible for impairment of LPS responsiveness and plasma membrane expression.** *A*, the LPS stimulation assay was done for TLR4 (L815AL816A)-EGFP as in Fig. 2. The data were from three independent experiments. *Small bars* indicate 95% confidence intervals of the mean (*p* value for * are: TLR4 (WT)-EGFP/MD-2 (+), $p = 0.002$). *B*, TLR4 (L815AL816A)-EGFP expression in HEK293T cells was observed by laser confocal microscopy. *Bar*, 20 μm.

was reasonable to explore whether leucines 815 and 816 need to be adjacent to each other. We created five genotypes of single amino acid mutants of TLR4: TLR4 (K813A)-EGFP, TLR4 (L815A)-EGFP, TLR4 (L816A)-EGFP, and TLR4 (D817A)-EGFP. We excluded the amino acid at position 814 from the analysis, because the amino acid in position 814 of wild-type TLR4 is alanine. The amino acid sequence alignment of wild-type TLR4 and the single amino acid replacement mutants is shown in Fig. 5. DNA sequences were confirmed by sequencing.

As was done with truncation mutants, we measured NF-κB activity of wild-type TLR4-EGFP, TLR4 (K813A)-EGFP, TLR4 (L815A)-EGFP, TLR4 (L816A)-EGFP, and TLR4 (D817A)-EGFP in response to LPS stimulation. All mutants except TLR4 (L815A)-EGFP showed responsiveness to LPS stimulation with coexpression of MD-2 (Fig. 6*A*). Without MD-2, no genotype of TLR4-EGFP responded to LPS stimulation (Fig. 6*B*). LPS stimulation was performed in an identical manner as with truncation mutants.

We analyzed the subcellular distribution of single amino acid mutants of TLR4-EGFP with and without MD-2 coexpression by fluorescence microscopy. TLR4 (K813A)-EGFP and TLR4 (D817A)-EGFP showed a similar fluorescence pattern to the wild-type, which localized at the plasma membrane when coexpressed with MD-2. No genotypes of TLR4-EGFP localized on the plasma membrane without MD-2 (Fig. 7). The cells transfected with TLR4 (L815A)-EGFP coexpressed with MD-2 did not show plasma membrane fluorescent pattern. Also, TLR4 (L815A)-EGFP showed comparatively weaker fluoresce than other mutants, possibly due to lower expression of the protein. Fluorescence of TLR4 (L816A)-EGFP with MD-2 was ambiguous as for the plasma membrane expression. Some kind of membranous structure was observed in the cytoplasmic area, but the intensity of the plasma membrane green fluorescence was obscure. Together with the results from the LPS stimulation experiment, the leucines at positions 815 and 816 are considered to play important roles in signal transduction and/or subcellular distribution of TLR4.

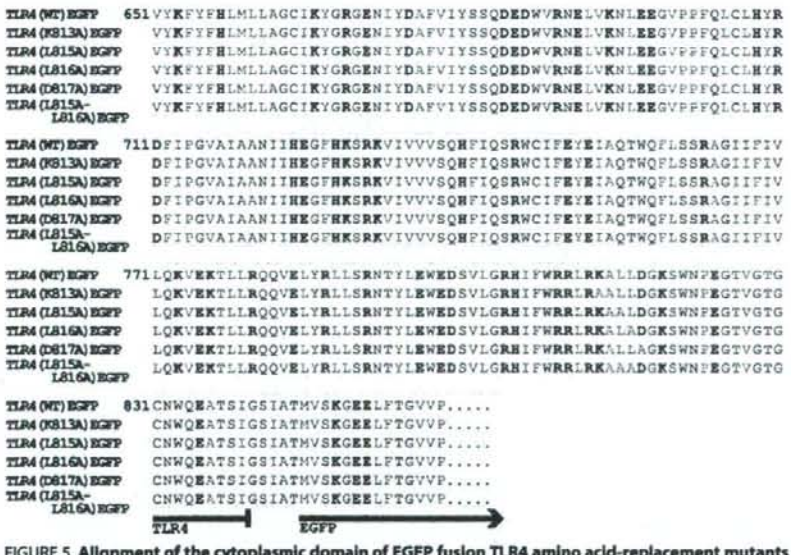

FIGURE 5. **Alignment of the cytoplasmic domain of EGFP fusion TLR4 amino acid-replacement mutants used in this study.** TLR4 (L813A) signifies a mutant with leucine replaced with alanine at position 813. Others are named in the same manner. The amino acids are *colored* as in Fig. 1. All amino acids are designated using the single-letter code.

Because EGFP consists of 239 amino acids, which is about one-third the size of the complete TLR4 protein, the experimental results obtained using TLR4-EGFP could have been influenced by the presence of the EGFP fused at the C terminus of TLR4. To rule out this possibility, we tested the functional integrity of both TLR4 (L815A) and TLR4 (L816A) with and without EGFP at the C terminus. Reporter assays were performed under the same conditions except that the shorter tag, FLAG-His_6, which has only 21-amino acid tags at the C terminus, was fused to TLR4 in place of EGFP. There was no difference

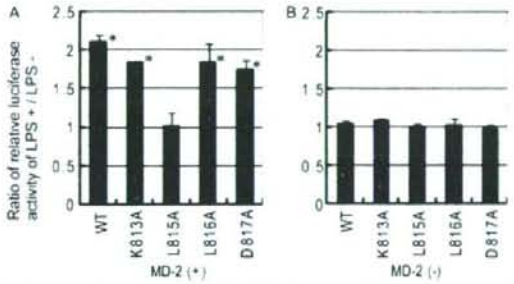

FIGURE 6. **Leucine at position 815 of TLR4 is pivotal for LPS responsiveness as measured by NF-κB luciferase assay.** *A*, HEK293T cells were transfected with single amino acid replacement mutants of the human TLR4-EGFP fusion protein plasmid, human MD-2 plasmid, and luciferase reporter and control plasmids. After 36 h, cells were stimulated with LPS (10 ng/ml) for 7 h, and luciferase reporter gene activity was measured. *B*, instead of MD-2, an empty vector was cotransfected with TLR4-EGFP plasmid and reporter assay vectors. LPS stimulation was done as in *A*. All results were expressed in the ratio of relative luciferase activity with LPS stimulation to that without the stimulation as in Fig. 2. The data were from three independent experiments. *Small bars* indicate 95% confidence intervals of the mean (p values for * are: TLR4 (WT)-EGFP/MD-2 (+), $p = 0.002$; TLR4 (K813A)-EGFP/MD-2 (+), $p = 0.000$; TLR4 (L816A)-EGFP/MD-2 (+), $p = 0.018$; and TLR4 (D817A)-EGFP/MD-2 (+), $p = 0.007$).

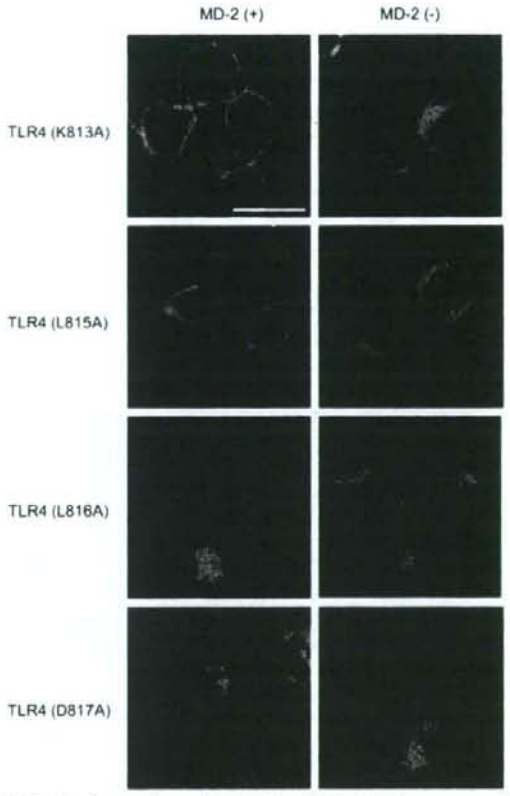

FIGURE 7. **Leucines at the position 815 and 816 of TLR4 are responsible for full plasma membrane expression.** Cells were cultured on coverslips in 12-well plates and transfected as in Fig. 2. EGFP-tagged TLR4 was visualized by laser confocal microscopy. Each genotype of TLR4-EGFP was cotransfected with human MD-2 plasmid or empty vector. *Bar*, 20 μm.

between EGFP-tagged proteins and FLAG-His_6-tagged proteins in the relative pattern of responsiveness against LPS stimulation (Fig. 8*A*). Because CD14 is also important for LPS recognition by TLR4, we examined the effect of CD14 coexpression on the phenotypic changes of the mutants (17, 18). Coexpression of CD14 did not change the phenotypes of wild-type TLR4, TLR4 (L815A), and TLR4 (L816A) in terms of LSP responsiveness (data not shown).

Cell surface expressions of the wild-type, L815A mutant, and L816A mutant TLR4-FLAG- His_6 fusion proteins were also examined. Live cells transfected with wild-type TLR4, the L815A mutant or the L816A mutant as well as human MD-2 and CD14 were biotinylated on the cell surface, and the biotinylated proteins were affinity-purified and subjected to Western blotting. Fig. 8*B* shows the marked difference in cell surface expression of wild-type and mutants L815A and L816A. Note that biotinylated proteins have additional residues on every amine of the extracellular domain, which leads to a band shift during electrophoresis. Although both mutants were detected far less than the wild-type on the cell surface, comparatively more L816A mutant was expressed on the plasma membrane than L815A mutant, and the amount of L815A mutant seemed to be negligible compared with the wild type. These results may clarify the ambiguity of the microscopic observation of TLR4 (L815A) and TLR4 (L816A). Plasma membrane expression of TLR4 was impaired when the leucine at 815 or 816 was replaced to alanine. But the leucine at 815 is more critical, and the mutant L816A may show the weaker phenotypic change.

To further investigate the characteristics of the TLR4 (L815A) mutant, we performed an immunoprecipitation assay of wild-type and mutant TLR4. Cells were transfected with a human MD-2-FLAG-His_6 expression vector and either the wild-type or the mutant (L815A) TLR4-EGFP expression vector. Anti-TLR4 monoclonal antibody (clone HTA125), anti-GFP polyclonal antibody, or anti-FLAG monoclonal antibody was added to the lysate and precipitated with Protein G-Sepharose beads. Collected proteins were eluted and subjected to Western blotting. The results are shown in Fig. 8*C*. TLR4 (L815A) was not immunoprecipitated with anti-TLR4 antibody (HTA125). HTA125 antibody was raised against TLR4-expressing cells (9) and recognizes the extracellular portion of TLR4. This result suggests that the amino acid replacement at position 815 may cause a change in the extracellular portion of TLR4 and/or that the replacement may also inhibit cell surface expression of the mutant protein. On the other hand, both wild-type TLR4-EGFP and mutant TLR4-EGFP were immunoprecipitated with anti-GFP polyclonal antibody, which recognized EGFP. However, of the two bands of TLR4, the heavier band seems to be somewhat faint in the mutant, whereas in the wild type the heavier band is at least as dense as the lighter one. TLR4 can be detected as two separate bands in a Western blot (19), especially under transient transfection conditions. The difference in proportion of the heavy and light bands between wild-type and mutant TLR4 may suggest that there is some difference in glycosylation. Furthermore, wild-type TLR4 was coprecipitated with MD-2-FLAG-His_6, but the mutant TLR4 could not be detected (Fig. 8*C*, *lanes 4* and *8*). Because MD-2 is